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Two Separate Mechanisms of T Cell Clonal Anergy to Mls -1^{a1}

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ABSTRACT. T cell tolerance to superantigen can be mediated by clonal anergy in which Ag-specific mature T cells are physically present but are not able to mount an immune response. We induced T cell unresponsiveness to minor lymphocyte stimulations locus antigen (Mls)-1^a in mice transgenic for TCR Vβ8.1 in three different systems: 1) injection of Mls-1^a spleen cells, 2) mating with Mls-1^a mice, and 3) bone marrow (BM) chimeras in which Mls-1^a is present only on nonhematopoietic cells. CD4$^+$8$^-$Vβ8.1$^+$ cells from all these groups did not proliferate in response to irradiated spleen cells from Mls-1^a mice. We compared the response of these cells by T cell/stimulator cell conjugate formation, Ca^{2+} mobilization, and proliferation assays. The mechanisms underlying the unresponsiveness of these T cells appear to differ. CD4$^+$8$^-$Vβ8.1$^+$ cells from Mls-1^a spleen cell-injected mice mobilized cytoplasmic Ca^{2+} but proliferated at a reduced level in response to cross-linking with anti-TCR mAb. However, these cells formed conjugates, mobilized Ca^{2+}, and proliferated in response to Mls-1^a when activated B cells were used as stimulators, although they produced reduced levels of IL-2. In Mls-1$^{a/b}$ Vβ8.1 transgenic mice, a subset in CD4$^+$8$^-$Vβ8.1$^+$ cells did not mobilize cytoplasmic Ca^{2+} after TCR cross-linking. Their conjugate formation, Ca^{2+} mobilization, or proliferation in response to Mls-1^a on activated B cells was undetectable. Finally, CD4$^+$8$^-$Vβ8.1$^+$ cells from the BM chimeras proliferated to TCR cross-linking at a partially reduced level and formed conjugates, mobilized Ca^{2+}, and proliferated in response to Mls-1^a on activated B cells. These features suggest that the mechanisms underlying the maintenance of anergy in Mls-1^a spleen cell-injected mice are distinct from those in Mls-1^a mice. *Journal of Immunology*, 1993, 151: 6062.

The immune system discriminates between foreign and self-Ag. This process, self-tolerance, is acquired in an individual during the development of the immune system and is brought about by inactivation or death of self-Ag specific lymphocytes. The proposed mechanisms underlying self-tolerance include clonal deletion, clonal anergy, and active suppression (1). Direct evidence for these mechanisms became available after the linkage between TCR Vβ region and the reactivity of these Vβ-expressing T cells to endogenous superantigens, such as Mls[5] was discovered (2–6). Using antibodies specific for

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[5] Abbreviations used in this paper: Mls, minor lymphocyte-stimulating locus antigen; BM, bone marrow; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; indo-1 AM, indo-1 acetoxymethyl pentaester; PE, phycoerythrin; DiIC22(3), 1,1'-didocosanyl-1-3,3,3',3' tetramethylindocarbocyanine perchorate.

the Vβ regions, it was demonstrated that self-reactive T cells are deleted during development in the thymus. These studies in conjunction with TCR transgenic mouse systems (7, 8) established that self-reactive T cells can be physically eliminated during their development in the thymus.

Evidence for clonal anergy as one of the mechanisms of T cell tolerance has been shown in several systems including mice expressing transgenic MHC in extrathymic sites only (9, 10), BM chimeras (11, 12), neonatally thymectomized mice (13), or Ag-injected animals (14). Intravenous injection of spleen cells from Mls-1^a mice into Mls-1^b mice induces the expansion of T cells expressing Vβ6 TCR during the first 3 to 4 days after injection, followed by disappearance of these cells (15). However, not all $V\beta6^+$ T cells are eliminated and some of them remain in the periphery. These cells do not proliferate in response to Mls-1^a and it has been proposed that they are anergic (14). Similarly, T cell anergy can be demonstrated in BM chimeras (11, 12, 16). When Mls-1^a mice are reconstituted with BM cells from Mls-1^bI-E^- mice, the resulting chimeras retain a substantial number of $V\beta6^+$ T cells in the periphery. However, these cells proliferate poorly to Mls-1^a stimulator cells and to cross-linking with anti-TCR Vβ6 mAb, suggesting that they are anergic to TCR occupancy.

After occupancy of the TCR with ligand on an APC or with anti-TCR antibody, the signal is transduced by a number of intracellular biochemical changes, which include protein phosphorylation and an increase in $[Ca^{2+}]_i$, which trigger diverse intracellular processes culminating in cell proliferation and effector function (17). Studies using T cell clones in vitro suggested $[Ca^{2+}]_i$ plays an important role in the induction of T cell anergy (18). The removal of free Ca^{2+} during the induction of clonal anergy prevented T cells from becoming anergic. Also, calcium ionophore alone induced unresponsiveness to subsequent stimulation with Ag. Furthermore, defective calcium mobilization in anergic T cells in response to TCR cross-linking was reported using TCR Vβ8.1 transgenic mice (19).

In many *in vivo* studies, T cell clonal anergy was defined as a defect in proliferation in response to mAb specific for TCR V region (11, 12, 16). However, little is known regarding the biochemical events underlying the defect in the proliferation of anergic cells. Herein, a TCR Vβ8.1 transgenic mouse model (20) was used to investigate the mechanisms of T cell unresponsiveness to Mls-1^a. Vβ8.1 transgenic mice express Vβ8.1 TCR on >95% of T cells. Tolerance induction to Mls-1^a in these mice renders most $CD4^+$ cells unresponsive to this Ag. Thus, molecular events in anergic T cells can be monitored without extensive purification steps. We set up three different systems in which $CD4^+8^-V\beta8.1^+$ T cells were present in the periphery but were unable to proliferate in response to Mls-1^a. The results suggested that at least two distinct forms of T cell anergy exist. One type of anergic T cell is able to mobilize cytoplasmic Ca^{2+} in response to anti-TCR mAb. This type of cells can form conjugates with stimulator cells, mobilize Ca^{2+}, and proliferate in response to Mls-1^a when activated B cells are used as stimulator cells. The other type of anergic T cell appears totally unresponsive to TCR occupancy.

Materials and Methods

Mice

The generation of TCR Vβ8.1 transgenic mice has been described previously (20). The transgenic mouse lines 21 and 2 were used. Both lines express transgenic TCR Vβ8.1 on >95% of mature T cells. We have not found any difference between these two transgenic lines. Line 21 was backcrossed to CBA/CaH (H-2^k, Mls-1^b) to produce Mls-1^bH-2^k Vβ8.1 transgenic mice. Line 2 was backcrossed to C57BL/6 (B6, H-2^b, Mls-1^b). Each neonate was examined for transgene integration by the polymerase chain reaction using Vβ8.1 and Jβ2.3 primers. Transgenic mice (after more than 7 generations of backcrossing) were used between 8 and 16 wk of age. CBA/CaH, CBA/J, and B6 mice were obtained from the National Cancer Institute (National Institutes of Health, Bethesda, MD). (B6xCBA/J)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (B6xCBA/CaH)F_1 mice were generated within the mouse facility of the University of Pennsylvania.

Preparation of BM chimeras

Recipient mice were exposed to 1100 rad from a ^{137}Cs source. After 6 to 8 h, irradiated mice were injected i.v. with $1–2 \times 10^7$ anti-Thy-1 mAb plus complement-treated BM cells from CBA/CaH or B6 Vβ8.1 transgenic mice via the tail vein. Some mice received a second dose of irradiation (900 rad) and were reconstituted by T cell-depleted BM cells 2 to 3 mo after initial BM transfer. Chimeras were maintained on antibiotics (polymyxin B and neomycin sulfate) in the drinking water for 2 to 4 wk after BM reconstitution. They were used within 2 to 6 mo of reconstitution.

mAb

The following mAb were used for this study: anti-Thy-1.2 (30-H12, ref. 21); anti-CD8 (53–6.7, ref. 22; or NEI-004, New England Nuclear, Boston); anti-CD4 (GK1.5, ref. 23; or RL172.4, ref. 24); anti-TCR Vβ8.1/8.2 (MM 5-2, ref. 25; or KJ16, ref. 26); anti-TCR Vβ8 (F23.1, ref. 27); anti-TCR Vβ8.2 (F23.2, ref. 3); anti-TCR Vα2 (B20.1, ref. 28); anti-TCR Vα8 (KT50, ref. 29); and anti-IL4 (11B11, ref. 30).

Injection of spleen cells

T cell-depleted spleen cells were prepared by treatment of CBA/J spleen cells with anti-Thy-1 mAb (30-H12) and

complement. The 1–2 × 10^7 treated cells were injected i.v. into Vβ8.1 transgenic mice via the tail vein.

Immunofluorescence

Nylon wool nonadherent spleen cells were stained with mAb in PBS containing 0.5% BSA and 0.1% sodium azide. Cells (5 × 10^5) were incubated in 100 μl staining medium containing hybridoma supernatant for 20 min, followed by FITC-conjugated anti-rat Ig for an additional 20 min. After three washes, a 10-fold excess of rat Ig was added to block unoccupied sites of the second antibody. In some staining, FITC anti-CD8 mAb was used. Cells were incubated with PE anti-CD4 mAb (Becton Dickinson, San Jose, CA) and biotin anti-Vβ8.1/8.2 mAb (MM 5-2, PharMingen, San Diego) for 20 min then washed and incubated with streptavidin-conjugated Tricolor (Caltag, San Francisco) for an additional 20 min. After three washes, cells were analyzed using a FACScan (Becton Dickinson).

Cell preparation and culture

Single cell suspensions were prepared from spleen. Spleen cells were applied to nylon wool columns and nonadherent cells were collected. In general, the proportion of T cells in the preparation was >80%. Enrichment of $CD4^+CD8^-$ T cells was performed using Dynabeads M-450 (Dinal Inc., Oslo, Norway). Nylon wool nonadherent spleen cells were incubated with anti-CD8 (53–6.7) mAb. After washing, cells were further incubated with anti-rat Ig-coated Dynabeads for 30 min at 4°C with constant agitation, and the bound population removed by magnetic equipment. After enrichment, $CD4^+$ cells were ~90% and $CD8^+$ cells were <2.5% except cells from Mls-$1^{a/b}$ transgenic mice. The CD8-depleted cell preparation of Mls-$1^{a/b}$ transgenic mice contained 50 to 60% $CD4^+CD8^-$ cells. The remaining were $CD4^-CD8^-$ cells, which contained ~30% Vβ8.1 TCR^+ cells and non-T cells (data not shown).

$CD4^+V\beta8.2^-$ cells were purified by cell sorting. Nylon wool nonadherent spleen cells of Mls-1^b or Mls-$1^{a/b}$ Vβ8.1 transgenic mice were stained in two colors with anti-TCR Vβ8.2 mAb (F23.2) plus FITC anti-mouse Ig and PE anti-CD4 mAb. Cells were incubated with F23.2 antibody for 20 min then washed and incubated with FITC anti-mouse Ig for an additional 20 min. After washing, mouse Ig (500 μg/ml) was added to block unoccupied sites of the second antibody. Cells were incubated with PE anti-CD4 mAb (Becton Dickinson) for 20 min then washed and the $CD4^+V\beta8.2^-$ population was sorted using $FACStar^{plus}$ (Becton Dickinson).

Stimulator cells were prepared by treating spleen cells from inbred mice with anti-Thy-1 mAb and complement followed by irradiation at 2000 rad. In some experiments, spleen cells were prepared from mice given 200 μl of goat anti-mouse IgD antiserum (anti-δ antibody) 24 h before death. These cells were irradiated at 3000 rad. Cultures were performed in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, 5 × 10^{-5} M 2-ME, 1% penicillin/streptomycin, and 10% heat-inactivated FCS. T cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for 2 days when anti-δ-treated B cells were used as stimulator cells, and for 4 days when spleen cells from nontreated mice were used as stimulator cells. Proliferation was assessed after 6 or 16 h exposure to 1 μCi [^{3}H]thymidine when anti-δ-treated or untreated stimulator cells were used, respectively. The results were expressed as the difference of mean counts between experimental and control responses of triplicate cultures including the SD of the mean.

Proliferative responses of T cells to anti-TCR mAb was performed using mAb KJ16. The supernatant from the KJ16 hybridoma was precipitated with ammonium sulfate then resuspended in ~1/50 vol and extensively dialysed in PBS. A culture plate was coated with 10 μg/ml goat anti-rat IgG (Sigma, St. Louis) in Tris base buffer (20 mM, pH 10) at 4°C for 5 to 16 h. After washing, wells were incubated with mAb KJ16 for 4 h at 4°C then washed and used for assays. Responder cells (5 × 10^4) were cultured for 2 days and proliferation was assessed after 6 h exposure to 1 μCi [^{3}H]thymidine.

Measurement of IL-2 production

IL-2 activity in the supernatant of in vitro cultured T cells was determined using IL-2/IL-4-responsive HT-2 cells. T cells from Vβ8.1 transgenic mice (4 × 10^5) were cultured with CBA/CaH or CBA/J T-depleted spleen cells from anti-δ antibody-treated mice (4 × 10^5). Supernatant from the culture was removed 1 day later. Serial dilutions of the supernatant were added to HT-2 cells (5 × 10^3). HT-2 cells were cultured for 2.5 days, and the response was measured using the MTT assay (31). The response of HT-2 cells was not inhibited by saturating concentrations of the anti-IL-4 mAb (11B11) (30), indicating that the factor responsible for HT-2 cell growth was IL-2. One unit of IL-2 was defined as the concentration of IL-2 required to support the half-maximal growth of HT-2 cells over the culture period.

Analysis of $[Ca^{2+}]_i$ after cross-linking with anti-TCR mAb

Nylon wool nonadherent T cell-enriched spleen cells were loaded with 1 μM indo-1 AM (Molecular Probes, Inc., Junction City, OR) for 30 min at 37°C then washed and resuspended in serum-free medium. Indo-1-loaded T cells were stained with PE-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-Vβ8.2 (F23.2), and biotin-conjugated anti-Vβ8.1/8.2 (KJ16) mAb for 20 min at 20°C. After washing, cells were resuspended in serum-free medium (37°C) and

analyzed using a Coulter Elite (Coulter, Hialeah, FL). Streptavidin-conjugated Cy-chrome (PharMingen) was added to cross-link Vβ8.1/8.2$^+$ TCR. PE$^+$FITC$^-$Cy-Chrome$^+$ (CD4$^+$Vβ8.1$^+$) cells were gated and their $[Ca^{2+}]_i$ was monitored by the ratio of absorbance at 525 and 395 nm (32).

Analysis of T cell/stimulator cell conjugate formation and $[Ca^{2+}]_i$ measurement

Flow cytometry analysis of T cell/stimulator cell conjugate formation and T cell $[Ca^{2+}]_i$ was performed as described previously (33). Briefly, nylon wool nonadherent spleen cells were loaded with indo-1 AM. Stimulator cells were prepared from mice injected with anti-δ antibody 24 h before death. Spleen cells were treated with anti-Thy-1 antibody plus complement then resuspended in 10% FCS containing RPMI1640 (2×10^7/ml) and stained with 3 μM DilC22(3) (Molecular Probes, Inc.) for 30 min at 37°C. T and stimulator cells were mixed then centrifuged and incubated for 5 min at 37°C. Cells were gently resuspended and analyzed by a modified FACS II (Becton Dickinson Immunocytometry System, Sunnyvale, CA) equipped with argon (model 2025, Spectraphysics, Mountain View, CA) and krypton (model 164-01, Spectraphysics) lasers. Indo-1 was excited by 100 mW all-band UV from the argon laser and DilC22(3) by 100 mW at 530 nm from the krypton laser. Indo-1 and DilC22(3) signals were detected simultaneously and all parameters were first gated by the indo-1 signal so that only indo-1-containing cells (T cells and T cell/stimulator cell conjugates) were detected. T cell/stimulator cell conjugates were identified as the DilC22(3)-positive population in indo-1-gated cells. Percentage of binding was obtained by counting events within the DilC22(3)-positive area. Relative $[Ca^{2+}]_i$ increase was obtained from mean $[Ca^{2+}]_i$ of DilC22(3)-positive T cells subtracted by the mean value of the unbound population.

Results

Unresponsiveness of CD4$^+$8$^-$Vβ8.1$^+$ T cells to Mls-1^a in TCR Vβ8.1 transgenic mice injected with Mls-1^a spleen cells

Tolerance to Mls-1^a in Vβ8.1 transgenic mice was induced using the following three methods. The first method used i.v. injection of T cell-depleted spleen cells from CBA/J (Mls-1^a) mice into Mls-1^b Vβ8.1 transgenic mice. This method was shown to induce tolerance in peripheral mature T cells in inbred mice (14). One to 2 wk later, T cells were enriched from spleen cells of control and CBA/J-injected animals. Flow cytometry analysis of the T cell population revealed that CBA/J-injected transgenic mice contained ~30 to 50% CD4$^+$8$^-$ T cells, essentially all of which expressed TCR Vβ8.1 and CD4 at levels comparable to untreated control mice (Fig. 1). Although CD4$^+$ cells were only 30% of T cells in this particular experiment, the proportion of CD4$^+$ cells was similar to control mice in most experiments. The expression of TCR Vα among CD4$^+$8$^-$Vβ8.1$^+$ cells was also determined by flow cytometry. The ratio of CD4$^+$ T cells expressing TCR Vα2 or Vα8 was not changed after injection of Mls-1^a spleen cells. Next, the CD4$^+$8$^-$ populations were enriched and tested for proliferative response to Mls-1^a and to anti-TCR Vβ8.1/8.2 mAb (Fig. 2*A*). The proliferative response of CD4$^+$8$^-$Vβ8.1$^+$ cells from CBA/J-injected Vβ8.1 transgenic mice was markedly reduced after reexposure to Mls-1^a in vitro. Addition of an exogenous source of IL-2 was not able to reverse the reduced proliferation (data not shown). The proliferative response of the same population to anti-TCR mAb was also markedly reduced, indicating that most CD4$^+$8$^-$Vβ8.1$^+$ T cells from Mls-1^a-injected mice were anergic to TCR occupancy.

Response of T cells from Mls-1$^{a/b}$ Vβ8.1 transgenic mice

As a second method to induce tolerance in Vβ8.1 transgenic mice, we generated F_1 progeny between CBA/J (Mls-1^a) and Mls-1^b Vβ8.1 transgenic mice (Mls-1$^{a/b}$ Vβ8.1 transgenic mice). In these mice, T cells developed in the presence of self-Mls-1^a. The mice contained CD4$^+$8$^-$ T cells in the mature T cell pool, although their proportion was reduced to ~15% of peripheral T cells (Fig. 1). In addition, not all T cells expressed the transgenic TCR. Approximately 50% of CD4$^+$8$^-$ cells expressed the Vβ8.1 TCR. The remaining CD4$^+$8$^-$ cells expressed endogenous TCR β-chains. Among these cells, T cells expressing TCR Vβ8.2 were 7.4% of CD4 cells. This Vβ8.2$^+$ population was visualized in the right lower corner of the flow cytometry analysis due to the competition of anti-TCR Vβ8.2 mAb with anti-TCR Vβ8.1/8.2 mAb for binding the TCR during the staining procedure. In terms of the expression of the TCR Vα chains, the ratio of CD4$^+$8$^-$ cells expressing Vα2 or Vα8 TCR in Mls-1$^{a/b}$ mice were increased when compared with those of Mls-1^b Vβ8.1 transgenic mice, suggesting that the TCR α-chain use in this population was altered.

CD4-enriched populations of Mls-1$^{a/b}$ transgenic mice did not proliferate in response to Mls-1^a but proliferated to anti-Vβ8.1/8.2 mAb at a reduced level when compared with transgenic T cells from Mls-1^b mice (Fig. 2*A*). However, CD8-depleted T cell populations of Mls-1$^{a/b}$ mice contained ~50% CD4$^+$8$^-$ and ~50% CD4$^-$8$^-$ cells. Vβ8.1$^+$ cells were ~50% of CD4$^+$CD8$^-$ cells and were therefore only ~25% of the CD8-depleted T cell population. CD4$^+$8$^-$Vβ8.2$^+$ cells were present at ~3–4% of the total population. In addition, ~30% of CD4$^-$8$^-$ cells expressed Vβ8.1/8.2 TCR. Because CD4$^+$8$^-$Vβ8.2$^+$ and CD4$^-$8$^-$Vβ8.1/8.2$^+$ cells may not be responsive to Mls-1^a

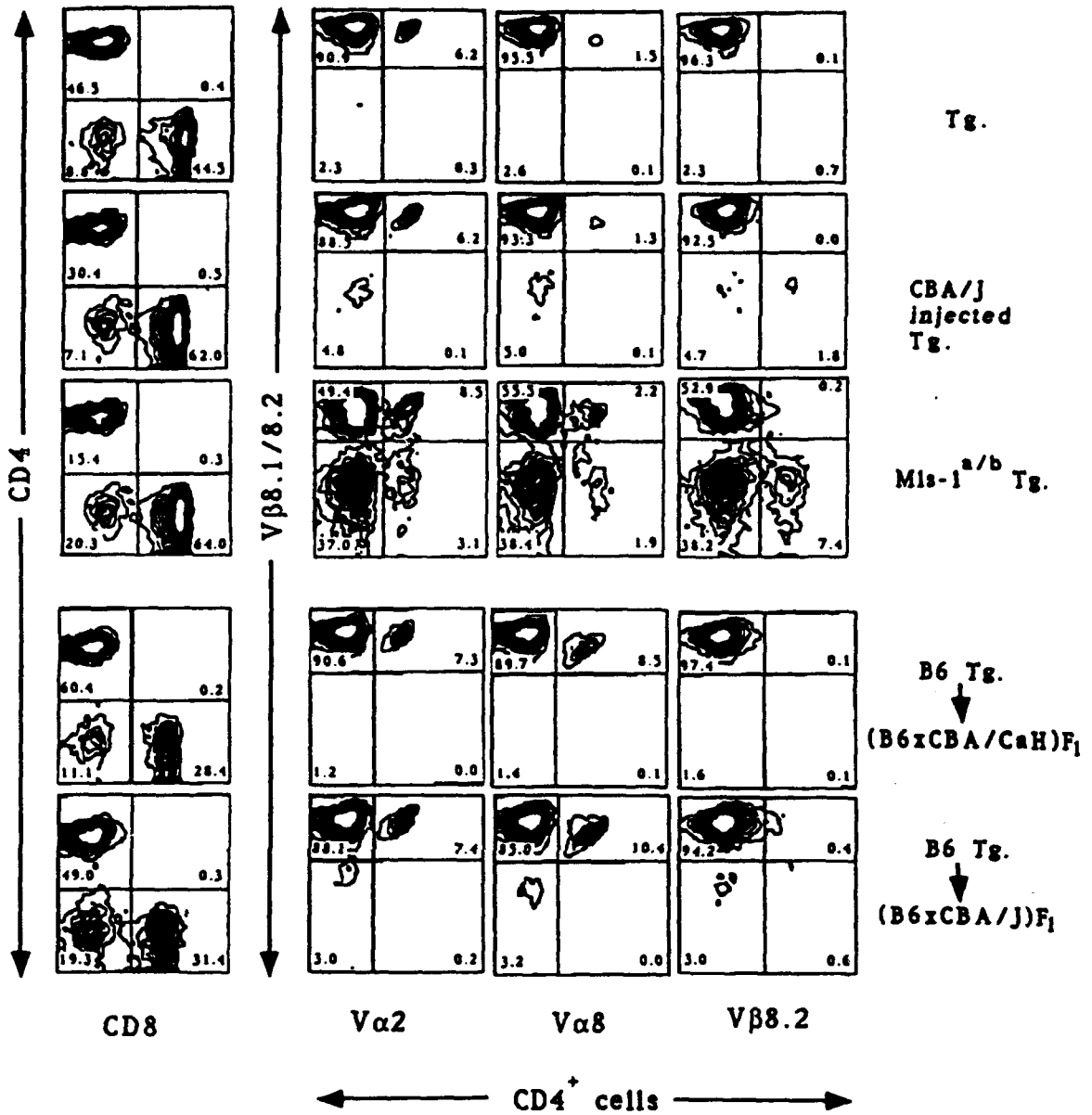

FIGURE 1. Expression of TCR, CD4, and CD8 on T cells tolerized to Mls-1^a. Nylon wool nonadherent spleen cells from a Mls-1^b Vβ8.1 transgenic mouse (Tg.), Mls-1^b transgenic mouse given T-depleted-CBA/J spleen cells (CBA/J injected Tg.), Mls-1$^{a/b}$ transgenic mouse (Mls-1$^{a/b}$ Tg.), B6 Tg.→(B6xCBA/CaH)F_1, and B6 Tg.→(B6xCBA/J)F_1 BM chimera were stained in two or three colors with FITC anti-CD8 (53–6.72) mAb or anti-Vα2 (B20.1) anti-Vα8 (KT50), or anti-Vβ8.2 (F23.2) mAb plus FITC anti-rat or FITC anti-mouse Ig antibody in addition to PE anti-CD4 mAb and biotin anti-Vβ8.1/8.2 mAb (MR5-2) plus streptavidin-Tricolor. In the *left panel*, staining profiles of total lymphocytes with CD4 and CD8 are shown. The percentage of cells falling into each quadrant is indicated. In the *right panel*, staining of TCR Vα2, Vα8, or Vβ8.2 vs Vβ8.1/8.2 mAb are shown after gating on the CD4$^+$ population. The number in *right upper* and *lower quadrants* is the percentage of cells determined after subtracting cells stained with second antibody alone. The Vβ8.2$^+$ population is seen in the *right lower corner* due to the *competition of anti-Vβ8.2 mAb with anti-Vβ8.1/8.2 mAb.*

and may not become tolerized, they might proliferate in response to anti-TCR Vβ8.1/8.2 mAb cross-linking. To eliminate these populations, we purified CD4$^+$8$^-$Vβ8.2$^-$ cells by cell sorting and analyzed their proliferative responses to anti-TCR Vβ8.1/8.2 mAb (Fig. 2*B*). The proliferative response of this population was 10 to 20% of the comparably sorted population from Mls-1^b transgenic mice. Approximately 50% of CD4$^+$Vβ8.2$^-$ cells express TCR Vβ8.1 in Mls-1$^{a/b}$ transgenic mice. After this adjustment is made, the proliferative response of CD4$^+$Vβ8.1$^+$ T cells from Mls-1$^{a/b}$ mice was approximately 20 to 40% of CD4$^+$Vβ8.1$^+$ cells in Mls-1^b mice.

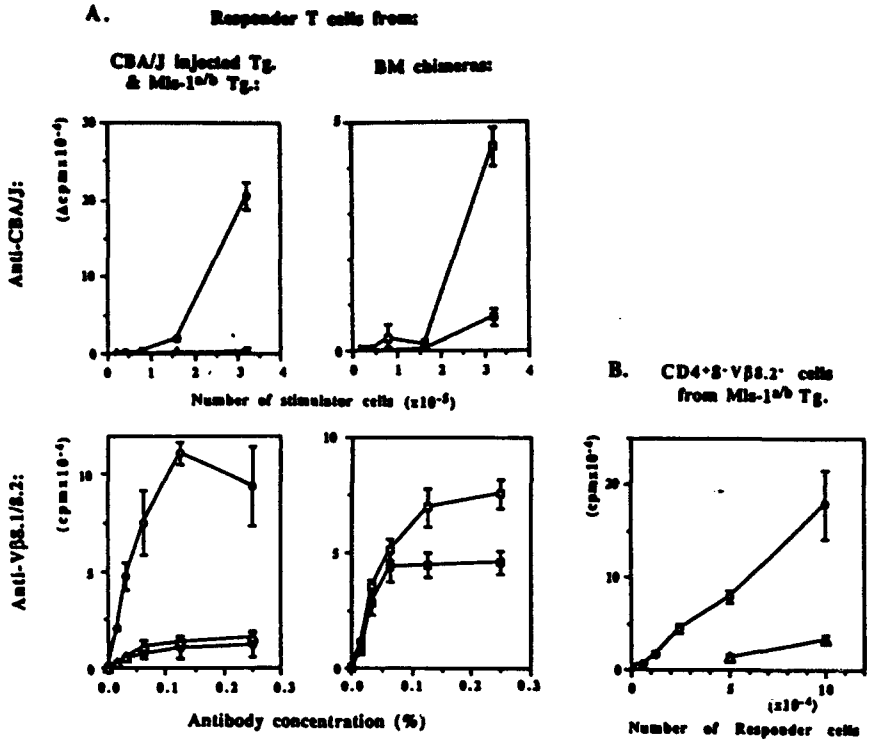

FIGURE 2. Proliferative responses of Vβ8.1⁺CD4⁺ T cells from CBA/J-injected transgenic, Mls-1$^{a/b}$ transgenic, or BM chimera mice to Mls-1^{a} or anti-TCR mAb cross-linking. *A*: CD4⁺CD8⁻ populations were enriched from nylon wool nonadherent spleen cells of control Mls-1^{b} (*open circle*), T-depleted CBA/J spleen cell-injected (*filled circle*), or Mls-1$^{a/b}$ (*open triangle*) Vβ8.1 transgenic mice in the *left panel*, or from those of B6Tg. →.(B6 × CBA/CaH)F_1 (*open square*) or B6Tg. → (B6 × CBA/J)F_1 (*filled square*) BM chimeras in the *right panel*. Nylon wool nonadherent spleen cells from each group were incubated with anti-CD8 (57–6.7) mAb and CD8⁺ cells were removed with anti-rat Ig-coated Dynabeads. The CD4-enriched T cells were incubated with irradiated T-depleted spleen cells of CBA/CaH or CBA/J or in the anti-Vβ8.1/8.2 (KJ16) mAb-coated plates. Cultures were maintained for 4 (anti-CBA/J) or 2 days (anti-KJ16) and the proliferation was assessed by 16 h (anti-CBA/J) or 6 h (anti-KJ16) exposure to [^{3}H]thymidine. Data are expressed as the difference of the mean cpm between experimental and control (anti-CBA/CaH) responses for anti-CBA/J responses (*upper panel*) or as mean cpm for anti-TCR responses (*lower panel*) and the SD of the mean. *B*: Proliferation of purified CD4⁺Vβ8.2⁻ cells from Mls-1^{b} (*open circle*) or Mls-1$^{a/b}$ (*open triangle*) Vβ8.1 transgenic mice in response to anti-TCR Vβ8.1/8.2 mAb was determined using sorted populations. Nylon wool nonadherent spleen cells of Mls-1^{b} or Mls-1$^{a/b}$ Vβ8.1 transgenic mice were stained in two colors with anti-TCR Vβ8.2 plus FITC anti-mouse Ig antibody and PE anti-CD4 mAb. CD4⁺Vβ8.2⁻ cells were purified by cell sorting using flow cytometry and placed in anti-Vβ8.1/8.2 mAb (KJ16) -coated plates. Cultures were maintained for 2 days and their proliferation was assessed by 6 h exposure to [^{3}H]thymidine.

Generation of BM chimeras that were reconstituted by BM cells from Vβ8.1 transgenic mice

We generated BM chimeras in which transgenic T cells developed in the presence of self-Mls-1^{a} on radioresistant element of the host cells in the absence of I-E⁺ hematopoietic cells. This method was shown to induce anergy of Mls-1^{a} reactive Vβ6⁺ T cells in normal mice, although H-2^{a} mice instead of H-2^{b} mice were used as BM donors in those experiments (11, 12, 16). For this purpose, (B6xCBA/J)F_1 (H-2$^{b/k}$Mls-1$^{b/a}$) or, as a control, (B6xCBA/CaH)F_1 (H-2$^{b/k}$Mls-1^{b}) mice were reconstituted with BM cells from transgenic mice backcrossed to C57BL/6 (B6 Tg.; I-E⁻ Mls-1^{b}) strain. Two months after 1100 rad irradiation and reconstitution with transgenic BM cells, the chimeras had 73 to 85% donor-derived T cells. Because this chimerism was not satisfactory for further studies of the transgenic BM-derived T cell population, we irradiated and reconstituted the hosts twice with transgenic BM cells with a total irradiation of 2000 rads (see *Materials and Methods*). These BM chimeras contained >95% donor-derived transgenic T cells. In B6 Tg.→ (B6xCBA/CaH)F_1 chimeras, in which Mls-1^{a} was absent, CD4⁺8⁻ cells were twice as abundant as CD4⁻8⁺ cells (Fig. 1). In contrast, the proportion of CD4⁺8⁻ cells was reduced in B6Tg.→ (B6xCBA/J)F_1 chimeras in which host Mls-1^{a} was present. In both chimeras, most CD4⁺8⁻ cells expressed Vβ8.1 TCR at equivalent levels. Interestingly, the proportion of Vα8⁺ cells in CD4⁺8⁻ cells was more than fivefold increased in both types of BM chimera when compared with Vβ8.1 transgenic mice. No significant difference in the

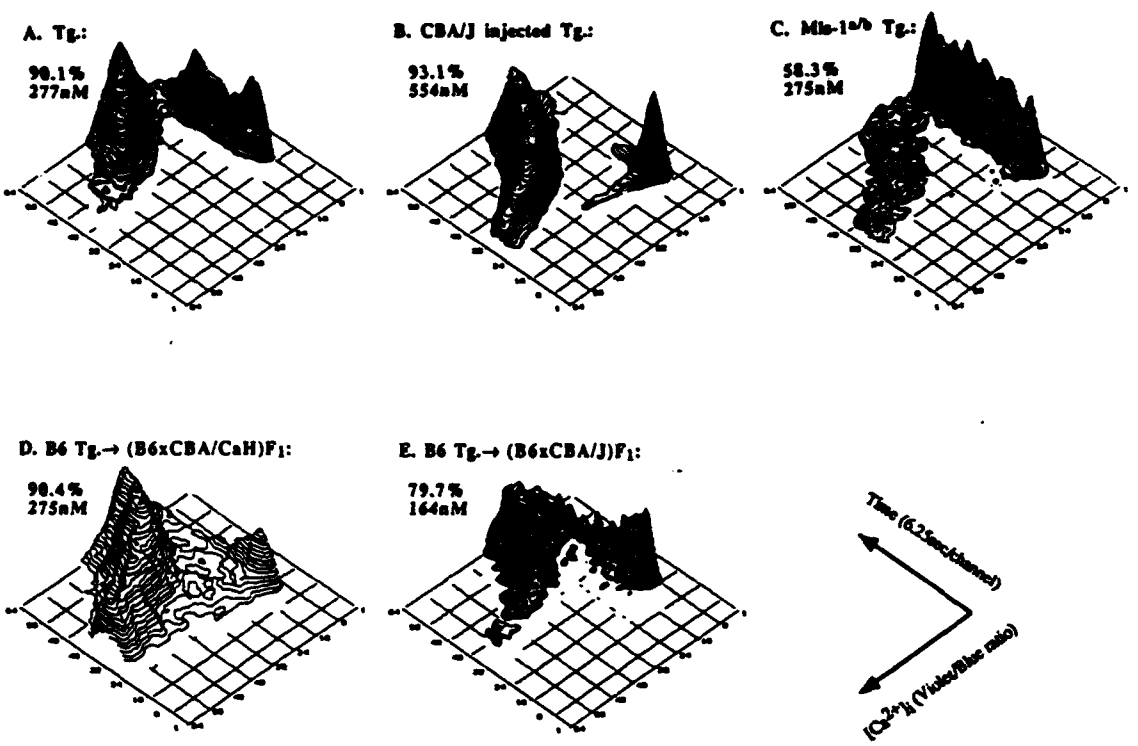

FIGURE 3. Mobilization of cytoplasmic-free Ca^{2+} in response to anti-TCR mAb in $CD4^+8^-$ Vβ8.1$^+$ cells from CBA/J-injected mice, Mls-1$^{a/b}$ transgenic mice, or BM chimeras. Nylon wool nonadherent spleen cells from Mls-1^b (Tg.), T-depleted CBA/J spleen cell-injected (CBA/J injected Tg.), or Mls-1$^{a/b}$ (Mls-1$^{a/b}$ Tg). Vβ8.1 transgenic mice as well as BM chimeras were loaded with indo-1 and were stained with PE anti-CD4, FITC anti-Vβ8.2 (F23.2), and biotin anti-Vβ8.1/8.2 mAb (KJ16). Flow cytometry measurement of indo-1 violet/blue fluorescence ratio was monitored after gating for PE^+FITC^-Cy-chrome$^+$ ($CD4^+$Vβ8.1$^+$) cells. Streptavidin-Cy-chrome was added to cross-link antibody-coated TCR Vβ8.1/8.2, and data collection was started at this point. Indo-1 violet/blue ratio measurements are only possible after streptavidin-Cy-chrome reaches cell surface bound biotin-KJ16. The upper number in each group refers to the percentage of cells that mobilized Ca^{2+} above background level. The base line was determined based on $[Ca^{2+}]_i$ of the resting ungated cells in each group. The lower number indicates the difference between the peak $[Ca^{2+}]_i$ of the responding cells and the mean of the base line $[Ca^{2+}]_i$.

ratio of Vα8$^+$ cells was observed between these two chimeras. CD4 cells expressing Vα2 were slightly increased in B6Tg.→(B6xCBA/J)F_1 chimeras.

The proliferative responses of CD4-enriched cells from BM chimeras were determined (Fig. 2*A*). $CD4^+8^-$Vβ8.1$^+$ cells from B6 Tg.→(B6xCBA/CaH)F_1 chimeras vigorously proliferated in response to Mls-1^a. In contrast, $CD4^+8^-$Vβ8.1$^+$ cells from B6 Tg.→(B6xCBA/J)F_1 chimeras proliferated minimally to the Ag, indicating that they were tolerant to Mls-1^a. However, the same population of CD4 cells proliferated in response to anti-TCR cross-linking. The level of proliferation was only ~35% reduced in CD4 cells from B6 Tg.→(B6xCBA/J)F_1 chimeras when compared with those from control chimeras. These data suggested that most $CD4^+8^-$Vβ8.1$^+$ cells in this chimera are able to proliferate in response to anti-TCR cross-linking, although they are unresponsive to Mls-1^a.

Mobilization of cytoplasmic Ca^{2+} on anti-TCR mAb cross-linking

To study early TCR-mediated signaling events in anergic T cells, we compared Ca^{2+} mobilization of CD4 cells after cross-linking with anti-TCR mAb. To determine $[Ca^{2+}]_i$ of $CD4^+8^-$Vβ8.1$^+$ cells, T cells were loaded with indo-1 AM and stained with anti-CD4 mAb and anti-TCR Vβ8.2 mAb. Although it has been reported that anti-CD4 mAb inhibited TCR-mediated increases in $[Ca^{2+}]_i$ (34), our preliminary studies indicated that the staining of T cells with PE anti-CD4 mAb had little effect on Ca^{2+} mobilization after anti-TCR mAb cross-linking in our assays. Staining with anti-Vβ8.2 mAb was used to distinguish Vβ8.1$^+$ cells from Vβ8.2$^+$ cells, because anti-Vβ8.1/8.2 mAb was used for cross-linking of the TCR. Cells were coated with biotinylated anti-TCR Vβ8.1/8.2 mAb, their TCR cross-linked by streptavidin-Cychrome, and $[Ca^{2+}]_i$ of $CD4^+8^-$Vβ8.1$^+$ cells was monitored (Fig. 3, Table I). In Vβ8.1 transgenic mice, >90% of CD4 cells mobilized cytoplasmic calcium after TCR cross-linking. Similarly, most CD4 cells from Mls-1^a-injected mice showed an increase in $[Ca^{2+}]_i$. The maximum increase in the level of $[Ca^{2+}]_i$ was higher than that of the control transgenic T cells and their response appeared accelerated. These data indicated that CD4 cells in Mls-1^a-injected mice mobilized cytoplasmic Ca^{2+} in response to TCR cross-linking but such biochemical

Table I
Mobilization of cytoplasmic Ca^{2+} in $CD4^+V\beta8.1^+$ cells after anti-TCR cross-linking

T Cell Source[a]	$CD4^+$ Cells Responding to anti-TCR mAb			
	Responding Cells[b](%)		Maximum $[Ca^{2+}]_i$ Increase[c] (nM)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Tg	90.1	91.6	277	398
Injected Tg	93.1	ND[d]	554	ND
Mls-$1^{a/b}$ Tg	58.3	43.4	275	151
Tg→(B6xCBA/CaH)F_1	90.4	91.5	275	222
Tg→(B6xCBA/J)F_1	79.7	63.6	164	101

[a] Nylon wool non-adherent spleen cells from each mouse were prepared as described in Figure 3. Graphic display of exp. 1 is shown in Figure 3.
[b] Percentage of $CD4^+V\beta8.1^+$ cells that increased $[Ca2+]_i$ above the level of resting cells after cross-linking with anti-TCR Vβ8.1/8.2 mAb (KJ16).
[c] The difference between the peak $[Ca^{2+}]_i$ of the responding cells and the mean of the base line $[Ca^{2+}]_i$.
[d] ND, not done.

change was not linked to proliferation. In contrast, the response of $CD4^+8^-V\beta8.1^+$ cells from Mls-$1^{a/b}$ transgenic mice was reduced and only half of them mobilized cytoplasmic Ca^{2+}. $CD4^+8^-V\beta8.1^+$ cells in Mls-$1^{a/b}$ transgenic mice appeared to contain two populations: one that mobilized Ca^{2+} and the other that did not in response to anti-TCR mAb cross-linking. Ca^{2+} mobilization of CD4 cells in BM chimeras was also tested. In B6 Tg.→(B6xCBA/CaH)F_1 BM chimera, >90% of $CD4^+8^-V\beta8.1^+$ cells mobilized cytoplasmic Ca^{2+}. A slightly reduced proportion of $CD4^+8^-V\beta8.1^+$ cells mobilized cytoplasmic Ca^{2+} in the B6 Tg.→(B6xCBA/J)F_1 chimera. The level of maximum Ca^{2+} increase was reduced in this chimera.

T cell/stimulator cell binding and cytoplasmic Ca^{2+} mobilization in response to Mls-1^a

We developed a system to measure T cell/stimulator cell conjugate formation and T cell $[Ca^{2+}]_i$ level simultaneously (33). This system enabled us to monitor early events in T cell activation with natural ligand, which involves coreceptors such as CD4 and adhesion receptors like LFA-1. Spleen cells from mice treated by prior injection of anti-δ antibody were used, because spleen cells from untreated mice were poor stimulators in this assay. Activation of B cells by in vivo treatment with anti-δ antibody induces an increase in the expression of B cell surface class II molecules and enhances the ability of B cells to stimulate T cells (35). Activated B cells also express higher levels of mammary tumor virus products (36), which may trigger up-regulation of Mls-1^a (37). When whole T cells from Vβ8.1 transgenic mice were tested using this system, 32.9% of them formed conjugates with Mls-1^a spleen cells and mobilized cytoplasmic calcium (Fig. 4). When separately analyzed, 52.6% of the $CD4^+8^-$ population bound and mobilized cytoplasmic calcium, whereas few of the $CD4^-8^+$ population responded.

We used this system to test responses of T cells from CBA/J-injected mice, Mls-$1^{a/b}$ Vβ8.1 transgenic mice, and BM chimeras (Fig. 5). The 29.8 and 28.2% of T cells from control and Mls-1^a-injected mice, respectively, formed conjugates with stimulator cells. Most cells responded with a similar rise in $[Ca^{2+}]_i$. A similar assay was performed using variable T cell stimulator cell dosages and we consistently found no difference between these two groups of T cells (data not shown). The data indicated that T cells tolerized to Mls-1^a by i.v. injection of Mls-1^a spleen cells were capable of responding to Mls-1^a stimulation by formation of conjugates with stimulator cells and a rise in the $[Ca^{2+}]_i$ level. These findings also support the notion that the repertoire of T cells was not altered with respect to their reactivity to Mls-1^a after injection of Mls-1^a spleen cells. In contrast, conjugate formation and Ca^{2+} mobilization was undetectable in T cells from transgenic mice of Mls-$1^{a/b}$ background. The estimated proportion of $CD4^+V\beta8.1^+$ cells in this T cell preparation was 7 to 8%. This assay can detect conjugate formation/Ca^{2+} increase of T cells from nontransgenic mice, in which Mls-1^a reactive (Vβ6, 7, 9, 8.1) CD4 cells are ~14% (3, 4, 38, 39). Although we think that the response of 7 to 8% of T cells in Mls-$1^{a/b}$ transgenic mice is in the detectable range, if present, it is also possible that the assay is not sensitive enough to detect Ca^{2+} increases in the few conjugates that could have taken place. The response of T cells from BM chimeras was also determined by the same assay system (Fig. 5*B*). T cells from B6 Tg.→(B6xCBA/J)F_1 chimeras bound and mobilized cytoplasmic-free calcium at levels indistinguishable from CBA/Ca transgenic or control BM chimeric T cells.

Proliferation of anergic T cells in response to Mls-1^a presented by activated B cells

The preceding studies showed that T cells from CBA/J-injected mice were able to form conjugates with Mls-1^a stimulator cells and increase $[Ca^{2+}]_i$. However, their proliferative response to irradiated spleen cells from Mls-1^a mice was greatly reduced. One of the possibilities to explain the lack of linkage between Ca^{2+} mobilization and T cell proliferation was that the stimulator cells were not identical. We used irradiated T cell-depleted spleen cells as stimulators for the proliferation assay, whereas we used spleen cells from mice treated by prior injection of anti-δ antibody for assays of cell/cell binding and $[Ca^{2+}]_i$ measurement. Thus, we compared proliferation of control and tolerized T cells against spleen cells from nontreated and anti-δ-treated mice (Fig. 6). Surprisingly, when anti-δ-treated B cells were used as stimulators, T cells from CBA/J-injected mice proliferated to Mls-1^a at levels similar to control T cells over a wide range of responder cell dosages. In addition, there was no significant difference in the dose

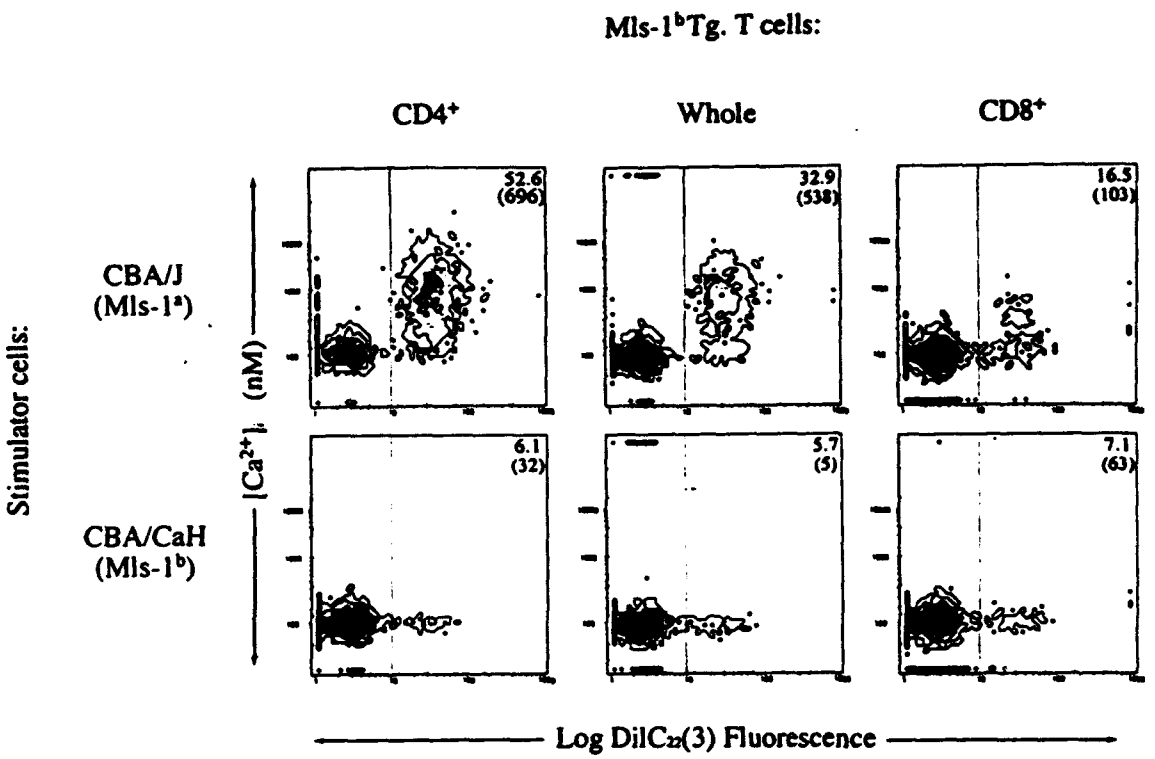

FIGURE 4. *Conjugate formation with stimulator cells and mobilization of cytoplasmic-free Ca^{2+} in Vβ8.1 transgenic T cells.* Nylon wool nonadherent spleen cells of Vβ8.1 transgenic mice (T cells) were treated with no antibody (whole), anti-CD8 mAb (NEI-004), or anti-CD4 mAb (RL172.4) plus complement followed by indo-1 loading. CD4-enriched population contained 83% $CD4^+8^-$ and <1% $CD4^-8^+$ cells. CD8-enriched population contained 86.7% $CD4^-8^+$ and <1% $CD4^+8^-$ cells. T-depleted spleen cells from goat anti-mouse δ antibody-treated mice were stained with DilC22(3) and used as stimulator cells. T and stimulator cells were mixed at a 1:4 ratio and analyzed by modified FACS II. *x*-axis indicates DilC22(3) fluorescence intensity that represents T cells/stimulator cell conjugate formation. T cells/stimulator conjugates were identified as the $DilC22(3)^+$ population. *y*-axis indicates the level of $[Ca^{2+}]_i$ of indo-1-loaded T cells. The numbers at the right upper corner indicate percentage of T cells binding to stimulator cells ($DilC22(3)^+$ cells/total indo-1^+ T cells). The numbers in the parentheses indicate relative $[Ca^{2+}]_i$ increase obtained from mean $[Ca^{2+}]_i$ of DilC22(3)-positive T cells subtracted by the mean value of unbound population.

of stimulator cells required to achieve half-maximal response of normal and anergic transgenic T cells (data not shown). However, production of IL-2 in response to Mls-1^a on activated B cells was reduced (Table II). This dose of IL-2 might have been sufficient to support the growth of anergic T cells in the proliferation assay.

In contrast, T cells from Mls-$1^{a/b}$ transgenic mice did not proliferate to anti-δ-treated stimulator cells. This unresponsiveness was not simply due to the reduced frequency of $CD4^+V\beta8.1^+$ cells in Mls-$1^{a/b}$ transgenic mice, because a $CD4^+$ cell-enriched population of Mls-$1^{a/b}$ mouse T cells, which contained ~20% $CD4^+V\beta8.1^+$ cells, did not proliferate in response to activated B cells from CBA/J mice (Fig. 7).

T cells from BM chimeras were also tested for their proliferation to anti-δ-treated stimulator cells. T cells from B6 Tg.→$(B6xCBA/J)F_1$ did not proliferate in response to conventional irradiated spleen cells of Mls-1^a mice. However, the same T cell population proliferated in response to Mls-1^a when presented by spleen cells from anti-δ-treated CBA/J mice at a level similar to those from control B6 Tg.→$(B6xCBA/CaH)F_1$ mice.

Discussion

$CD4^+8^-V\beta8.1^+$ T cells from Mls-1^a-injected mice proliferated at a reduced level not only in response to Mls-1^a but also to anti-TCR cross-linking, which suggested that this population was anergic to TCR-mediated stimulation. However, despite defective proliferative responses, these T cells were able to mobilize cytoplasmic free Ca^{2+} in response to anti-TCR cross-linking. These data suggest that the early components of the signaling pathway through TCR, which induce mobilization of cytoplasmic-free Ca^{2+}, are functionally intact, in agreement with the previous studies using anergic T cell clones (40, 41). Therefore, the reduced proliferative response of these T cells may be due to the cellular events subsequent to or independent of calcium mobilization. In addition, these T cells were able to form conjugates with Mls-1^a stimulator cells and mobilize

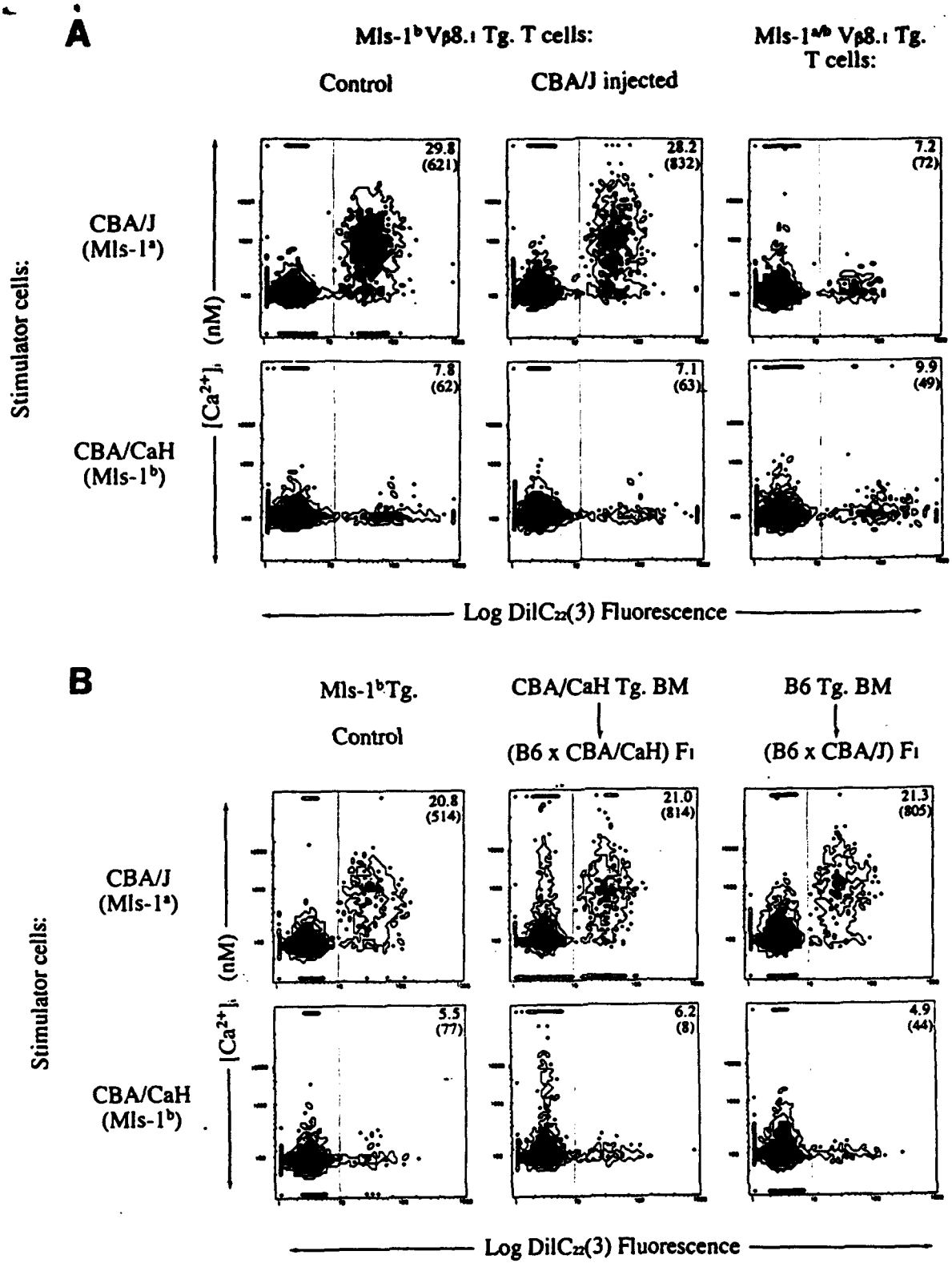

FIGURE 5. Conjugate formation and calcium mobilization of Vβ8.1+ T cells from CBA/J given transgenic mice and from BM himeras. *A*: Nylon wool nonadherent spleen cells from Mls-1b Vβ8.1 transgenic mice given PBS (control) or T-depleted CBA/J pleen cells (CBA/J injected) and Mls-1a/b Vβ8.1 transgenic mice were loaded with indo-1. Cells were mixed with DiI22(3)-abeled stimulator cells from anti-δ-treated mice at 1:2 ratio and analyzed using flow cytometry. Numbers at the right upper orners indicate percentage of binding. Numbers in parentheses indicate relative $[Ca^{2+}]_i$ increase obtained from mean $[Ca^{2+}]_i$ f DiIC22(3)-positive T cells subtracted by the mean value of unbound population. *B*: (B6 × CBA/CaH)F_1 or (B6 × CBA/J)F_1 ice were irradiated and reconstituted twice with T-depleted transgenic BM cells and used after 2 mo of second reconstitution hen the spleen T cells were >95% donor type. Nylon wool nonadherent spleen cells of control Vβ8.1 transgenic mice and vice irradiated BM chimeras were loaded with indo-1 and were analyzed as in *A*.

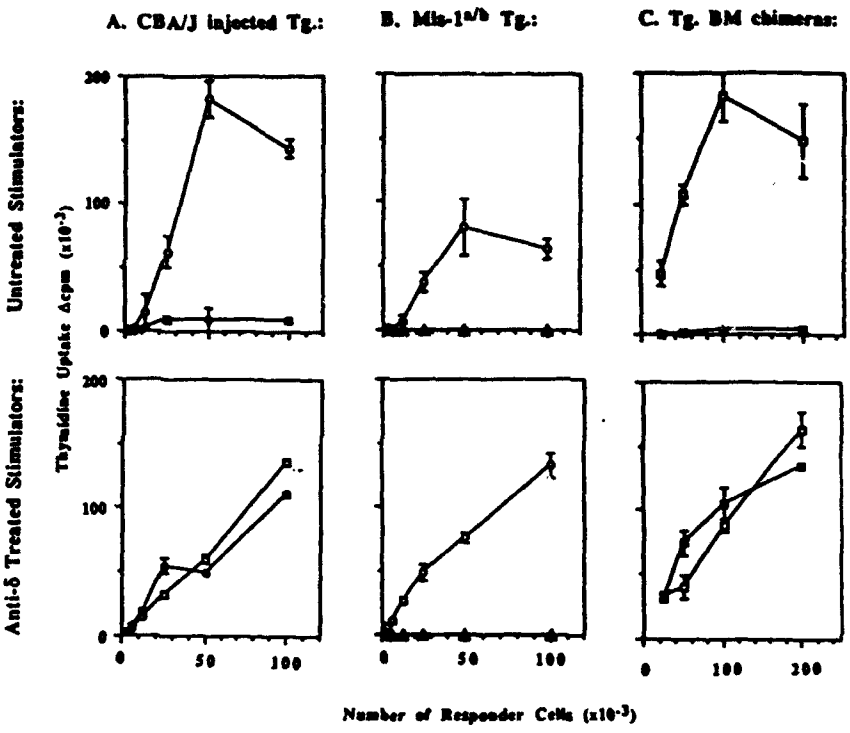

FIGURE 6. Proliferation of Vβ8.1 transgenic T cells in response to spleen cells from anti-δ-treated CBA/J mice. Nylon wool nonadherent spleen cells were prepared from control Mls-1^b (*open circle*), T-depleted CBA/J spleen cell-injected Mls-1^b (*filled circle*), Mls-$1^{a/b}$ (*filled triangle*) Vβ8.1 transgenic mice, or B6 Tg. → (B6 × CBA/CaH)F_1 (*open square*) and B6 Tg. → (B6 × CBA/J)F_1 (*filled square*) BM chimeras. Stimulator cells were prepared from spleen cells of untreated or anti-δ-injected CBA/CaH and CBA/J mice. T cells ($1 \times 10^5 - 3 \times 10^3$) were cultured with irradiated spleen cells (4×10^5) from CBA/CaH or CBA/J mice for 3 (anti-δ-treated stimulators) or 5 (untreated stimulator cells) days and proliferation was assessed after 16 h exposure to 1 μCi [^{3}H]thymidine. The data are expressed as the difference between experimental (anti-CBA/J) and control (anti-CBA/CaH) responses (Δcpm) and the SD of the mean.

Table II
Production of IL-2 in response to Mls-1^a on activated B cells

Responders	Production of IL-2 (U/ml) in Response to			
	Exp. 1		Exp. 2	
	CBA/CaH	CBA/J	CBA/CaH	CBA/J
Tg	<1	714	<1	476
Inj	<1	26	<1	15

T cells from untreated Vβ8.1 transgenic mice (Tg) or transgenic mice that had been inoculated with T-depleted CBA/J spleen cells (Inj) were stimulated with T-depleted CBA/CaH or CBA/J spleen cells from anti-δ antibody-treated mice. One day later, supernatant was removed and the IL-2 content was measured by MTT assay using HT-2 indicator cells. Proliferation of HT-2 cells was not inhibited by anti-IL-4 mAb (11B11) indicating that the growth factor was IL-2. Exp. 1 was performed using CD4-enriched T cells (~90% $CD4^+$) and exp. 2 was performed using nylon wool nonadherent spleen and lymph node cells.

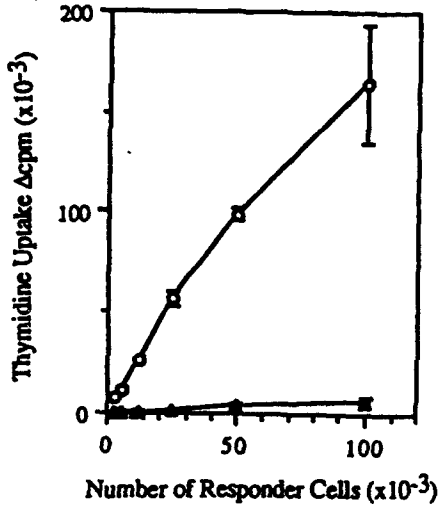

FIGURE 7. Proliferation of $CD4^+$-enriched cells of Mls-1^b and Mls-$1^{a/b}$ Vβ8.1 transgenic T cells in response to spleen cells from anti-δ-treated CBA/J mice. $CD4^+$ cells were enriched from nylon wool nonadherent spleen cells of control Mls-1^b (*open circle*) and Mls-$1^{a/b}$ (*filled triangle*) Vβ8.1 transgenic mice. Assay was performed as Figure 6. The proportions of $CD4^+V\beta8.1^+$ cells from Mls-1^b and Mls-$1^{a/b}$ mice were 84 and 19%, respectively.

cytoplasmic-free Ca^{2+} when stimulated by spleen cells from anti-δ-treated mice. These features suggest that the Mls-1^a recognition and initial binding of T cells is not altered after injection of Mls-1^a spleen cells. These cells remained defective in IL-2 production, whereas they were able to proliferate in response to Mls-1^a on activated B cells. It is unclear why anergic cells proliferated, despite reduced IL-2 production. The level of IL-2 produced might be sufficient for their proliferation. Alternatively, factors other than IL-2 or IL-4 might be involved in their proliferation.

Most peripheral $CD4^+8^-V\beta8.1^+$ T cells that populate the periphery of Mls-$1^{a/b}$ Vβ8.1 transgenic mice also did not proliferate in response to Mls-1^a or anti-TCR cross-linking, but the mechanisms underlying the unresponsiveness appear to be distinct from that of Mls-1^a-injected mice by several criteria. Consistent with an earlier study, a subpopulation of these cells did not mobilize cytoplasmic Ca^{2+} in response to TCR cross-linking, in sharp contrast to those in Mls-1^a-injected transgenic mice. Furthermore, they did not mobilize detectable level of cytoplasmic Ca^{2+} or proliferate in response to activated Mls-1^a spleen cells. The

lack of Ca^{2+} response of this T cell population suggests that the TCR-mediated signaling pathway in these cells may be modulated at an early phase of activation occurring before and being linked to Ca^{2+} mobilization. It is unclear how unresponsiveness of these T cells occurred. One possibility is that a population of CD4 cells is rendered anergic after encountering self-Mls-1^a due to a mechanism of self-tolerance. An alternative possibility is that a subpopulation of $CD4^+$ cells is unresponsive to Mls-1^a due to preexisting biochemical defects in TCR signal transduction, which enabled them to escape clonal deletion. Currently, we cannot distinguish between these possibilities. A similar reduction of Ca^{2+} mobilization in response to anti-TCR mAb was observed in CD4 cells in other Vβ8.1 transgenic mice (19). In their transgenic mice, >50% of $CD4^+8^-$Vβ8.1$^+$ cells remained in the periphery of Mls-$1^{a/b}$ mice. A subpopulation of these T cells were anergic to TCR occupancy and did not mobilize Ca^{2+} after TCR cross-linking. In contrast to these mice, ours have only ~10% of the $CD4^+8^-$ cells when compared with those in Mls-1^b Vβ8.1 transgenic mice and only half of them express transgenic TCR Vβ8.1. The difference in the gene construct of their and our transgenic mice is the sequence of Vβ8.1-Jβ2.3 junctional region, which may have resulted in the difference in the number of anergic T cells in Mls-$1^{a/b}$ mice. It would be interesting to find out whether similar phenotype can be seen in mature T cells of other TCR transgenic mice.

Although a subpopulation of $CD4^+8^-$Vβ8.1$^+$ cells in Mls-$1^{a/b}$ transgenic mice did not mobilize cytoplasmic Ca^{2+} in response to anti-TCR, others mobilized cytoplasmic Ca^{2+}. At least a portion of this population appear to proliferate in response to anti-TCR mAb, because 20 to 40% of proliferative response was observed in $CD4^+$Vβ8.1$^+$ cells of Mls-$1^{a/b}$ transgenic mice. They did not, however, proliferate in response to Mls-1^a. We speculate that this population may have reduced avidity to Mls-1^a and are not tolerized by self-Mls-1^a. We previously reported that alloreactive T cell clones that were isolated from Mls-$1^{a/b}$ Vβ8.1 transgenic mice by repeated stimulation with C57BL/6 alloantigens could proliferate in response to self-Mls-1^a, although at a reduced level (42). These clones required more Ag for their optimal proliferation and were much more sensitive to inhibition by anti-CD4 mAb, suggesting that the TCR on these clones have reduced avidity to Mls-1^a, despite Vβ8.1 TCR expression. These T cell clones may be derived from this subpopulation of CD4 cells that mobilized Ca^{2+} in response to anti-TCR mAb. The higher frequency of Vα2$^+$ or Vα8$^+$ T cells among $CD4^+8^-$ cells in our Mls-$1^{a/b}$ Vβ8.1 transgenic mice support this idea. It was reported that TCR α-chains influence the reactivity of Vβ8.1 T cells to Mls-1^a (43). Using T cell hybridomas isolated from their Vβ8.1 transgenic mice Smith et al. showed that Vα11$^+$ hybridomas are biased toward Mls-1^a reactivity, whereas Vα2$^+$ and Vα8$^+$ hybridomas are biased against Mls-1^a reactivity. Taken together, $CD4^+8^-$ Vβ8.1$^+$ cells in Mls-$1^{a/b}$ Vβ8.1 transgenic mice appear to contain two different types of cells. One population is anergic and cannot mobilize Ca^{2+} in response to TCR occupancy. The other population express Vβ8.1 TCR that has reduced avidity to TCR. This latter population is not tolerized and can respond to appropriate TCR occupancy.

In B6 Tg.→(B6xCBA/J)F_1 BM chimeras, in which T cells differentiate in I-E^+Mls-1^a environment in the absence of I-E^+ hematopoietic cells, $CD4^+8^-$Vβ8.1$^+$ cells appear to have mixed characters of those from Mls-1^a spleen cell-injected and Mls-$1^{a/b}$ transgenic mice. T cells in this BM chimera appear to contain anergic cells that resemble those in Mls-$1^{a/b}$ transgenic mice, because their Ca^{2+} mobilization in response to anti-TCR mAb was partially reduced. On the other hand, T cells in the chimera formed conjugates then mobilized cytoplasmic Ca^{2+} and proliferated in response to anti-δ-treated Mls-1^a spleen cells, which was reminiscent of T cells from Mls-1^a spleen cell-injected transgenic mice. T cells in the BM chimera may contain two different types of anergic cells. One set of T cells are similar to those in Mls-$1^{a/b}$ transgenic mice and the other type resembles those in CBA/J-injected transgenic mice. Alternatively, unresponsiveness of T cells in BM chimera to Mls-1^a may be mediated by a third type of mechanism that was not observed in Ag-injected or Mls-$1^{a/b}$ mice. The avidity of the TCR to Mls-1^a may also play a role on the unresponsiveness of these $CD4^+8^-$Vβ8.1$^+$ cells in the BM chimera. It is likely that TCR in B6 Tg.→(B6xCBA/J)F_1 chimera have reduced avidity to Mls-1^a, because these T cells proliferated to anti-TCR cross-linking at only a partially reduced level, whereas they proliferated minimally to Mls-1^a on irradiated spleen cells. These results suggest that the underlying mechanisms of T cell unresponsiveness in bone marrow chimeras are not necessarily identical to those of Ag-injected animals or Mls-$1^{a/b}$ mice. Studies are in progress to discriminate among these possibilities.

In summary, unresponsiveness of specific Vβ$^+$ T cells to the superantigens appears to occur by at least three distinct mechanisms. The first type of unresponsive T cells are found in a subpopulation of Mls-$1^{a/b}$ transgenic mice. The TCR on these cells may not have enough avidity for the Mls-1^a, despite the specific TCR Vβ expression, and cells may not receive activation signals. They can be activated by other appropriate ligands, and therefore are not functionally anergic. The second type of unresponsive T cells present in Mls-$1^{a/b}$ Vβ8.1 transgenic mice are anergic, because they do not proliferate in response to anti-TCR cross-linking. In these cells, early signaling events through TCR appear to be altered in such a way that TCR stimulation cannot mobilize cytoplasmic Ca^{2+}. The third type of unresponsive cells, which are found in superantigen-injected animals, are also anergic, but the molecular mechanisms

underlying their unresponsiveness are distinct from the former type. Although these anergic cells do not proliferate, they can mobilize cytoplasmic-free Ca^{2+} in response to TCR cross-linking. In addition, these anergic cells can form conjugates then mobilize Ca^{2+} and proliferate when activated B cells are used as stimulator cells. These two forms of anergy may be induced and maintained by distinct mechanisms and the consequence of the anergy may also differ. The latter type of anergic cells can proliferate under optimal conditions in vitro, suggesting that they might retain some of their functional abilities in vivo. In addition, a recent study by Rocken et al. (44) suggested that this type of anergy can be reversed after infection with the nematode *Nippostrongylus brasiliensis*. It will be critical to understand how these different types of anergy are involved in the induction and maintenance of self-tolerance, whereas sparing the ability of the immune system to protect from foreign invaders.

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